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Protein kinase CK2 inactivates PRH/Hhex using multiple mechanisms to de-repress VEGF-signalling genes and promote cell survival

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ABSTRACT

Protein kinase CK2 promotes cell survival and the activity of this kinase is elevated in several cancers including chronic myeloid leukaemia. We have shown previously that phosphorylation of the Proline-Rich Homeodomain protein (PRH/Hhex) by CK2 inhibits the DNA-binding activity of this transcription factor. Furthermore, PRH represses the transcription of multiple genes encoding components of the VEGF-signalling pathway and thereby influences cell survival. Here we show that the inhibitory effects of PRH on cell proliferation are abrogated by CK2 and that CK2 inhibits the binding of PRH at the Vegfr-1 promoter. Phosphorylation of PRH by CK2 also decreases the nuclear association of PRH and induces its cleavage by the proteasome. Moreover, cleavage of phosphorylated PRH produces a stable truncated cleavage product which we have termed PRHΔC (HhexΔC). PRHΔC acts as a transdominant negative regulator of full-length PRH by sequestering TLE proteins that function as PRH co-repressors. We show that this novel regulatory mechanism results in the alleviation of PRH-mediated repression of Vegfr-1. We suggest that the re-establishment of PRH function through inhibition of CK2 could be of value in treatment of myeloid leukaemias, as well as other tumour types in which PRH is inactivated by phosphorylation.

INTRODUCTION

Protein kinase CK2 (Casein Kinase II) is a serine/threonine protein kinase that functions to promote cell survival by regulating the activity of proteins involved in many processes in the cell including transcription, cell signalling, cell-cycle control and DNA repair (1–3). The active CK2 enzyme is a tetramer consisting of two catalytic α subunits and two regulatory β subunits that modulate kinase activity, substrate specificity and sub-cellular localization (2). CK2 activity is elevated in several cancer types (4) including Acute Myeloid Leukaemia (AML) and Chronic Myeloid Leukaemia (CML) (5,6). Phosphorylation by CK2 alters the activity and/or stability of the tumour suppressor proteins p53, PML and PTEN, changing their affinity for their respective targets and/or altering their degradation by the proteasome, ultimately leading to increased cell survival (1). CK2 activity also inhibits the degradation of several oncoproteins and other pro-survival proteins again leading to enhanced cell survival. Additionally CK2 has an anti-apoptotic role and inactivates a number of proteins involved in promoting apoptosis (1–3).

The Proline-Rich Homeodomain (PRH/Hhex) protein regulates many processes in embryonic development and in the adult [reviewed (7)]. In the haematopoietic system PRH is expressed in all myeloid lineages where it functions as a negative regulator of cell proliferation (8–10). PRH interacts with eIF4E and inhibits the mRNA transport of proliferation control mRNAs such as the cyclin D1 mRNA (8,11). PRH also interacts with the PML protein although the importance of this interaction in the control of cell proliferation is not known (11). Loss of PRH function in myeloid cells contributes to the development of AML subtypes and blast crisis CML (12,13). Outside the haematopoietic system, down-regulation and mislocalization of PRH is associated with thyroid cancer and breast cancer (14,15).

PRH is an oligomeric transcription factor that binds to tandem arrays of PRH-binding sites inducing significant DNA condensation (16,17). PRH can activate or repress

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the transcription of its target genes. One mechanism that PRH uses to repress transcription involves the recruitment of members of the TLE/Groucho family of co-repressor proteins (18). TLE co-repressors are recruited to promoters through interaction with a DNA-binding transcription factor, bind directly to non-acetylated histones and recruit histone deacetylases to bring about transcriptional repression (19). An Eh1 motif present in the N-terminal repression domain of PRH mediates the binding of PRH to TLE proteins and this motif is required for co-repression (18). We have shown that PRH regulates haematopoietic and breast cell survival through the direct transcriptional repression of multiple genes encoding components of the VEGF-signalling pathway (VSP) including Vegf, Vegfr-1, Vegfr-2 and neuropillin-1 (10,20). VEGF signalling is required for normal angiogenesis and haematopoiesis and elevated VSP activity is often associated with leukaemias and solid tumours, suggesting that deregulation of this pathway commonly occurs in tumourigenesis (21).

Our recent work showed that phosphorylation of PRH by CK2 inhibits the DNA-binding activity of this protein (20). Here we show that CK2 abrogates the inhibitory effect of PRH on the proliferation of haematopoietic cells and we reveal multiple additional mechanisms through which the phosphorylation of PRH leads to the inhibition of PRH activity and the up-regulation of VEGF-signalling genes.

MATERIALS AND METHODS

Expression plasmids

pMUG1-Myc-PRH expresses human PRH tagged with the Myc9E10 epitope (18). pMUG1-Myc-PRH S163E,S177E was described previously (20). pMUG1-Myc-PRH S163C,S177C, pMUG1-Myc-PRH S163E,S177E Δ211 and pMUG1-Myc-PRH S163E,S177E Δ211 F32E were created using a Quikchange mutagenesis kit according to the manufacturer's instructions. pRc/CMV-CK2α-HA, pRc/CMV-HACK2β and pRc/CMV-CK2a-K68M-HA express HA-tagged CK subunits and a kinase-dead CK2α mutant respectively and were a gift from Professor D. Litchfield (University of Western Ontario). The plasmid expressing FLAG tagged TLE1 was a gift from Professor S. Stifani (McGill University) and has been described previously (18).

Cell culture, transient transfections and knockdown experiments

K562 cells were obtained from Professor C. Bunce (University of Birmingham) and originally purchased from ATCC. K562 cells were checked for glycophorinA expression using PCR and antibodies. Cell culture and transient transfections were performed as described previously using equal amounts of total DNA in each case (18,22). PRH knockdown (PRH KD) cells were produced as described previously (10).

Co-immunoprecipitation assays and western blotting

Co-immunoprecipitation assays were performed exactly as described previously (18,22). An amount of 20 μg of cell extract was used in western blotting experiments with PRH and phosphorylated PRH (pPRH) antibodies (16).

Inhibitor experiments

Control or PRH shRNA KD cells were incubated with 80 μM DMAT (2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole, Calbiochem) for 24 h. Protein stability experiments were performed with K562 cells treated with 40 μM Anisomycin (Sigma) for 4, 8 or 24 h. Proteasome activity was inhibited with 10 μM MG132 (Sigma) for 4, 8 or 24 h.

In situ cell fractionation and biochemical fractionation

In situ cell fractionation was performed as described previously (23). Briefly, poly-L-transfected K562 cells were plated onto lysine coated microscope coverslips. Cytoplasmic and loosely held nuclear proteins were removed as required using CSK buffer (10 mM PIPES, 300 mM Sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA). Untreated cells and extracted cells were fixed in 4% parafomaldehyde for 30 min, rinsed with PBS and incubated with PBS + 3% BSA for 40 min. The cells were then incubated with either rabbit anti-HA polyclonal antibody (Sigma), rabbit anti-Myc9E10 antibody (Cell Signalling), rabbit Lamin B antibody (Santa Cruz Biotechnology) or a mouse Tubulin antibody (Sigma) and the appropriate secondary antibodies (Stratec). Coverslips were mounted with DAPI (4′,6-diamidino-2-phenylindole)-containing mounting medium (Vectashield) and viewed on a Leica DM IRBE confocal microscope. Imaging was performed using Leica Confocal Software Version 2.00.

Biochemical fractionation for western analysis was performed as described previously (22). Briefly, whole-cell extracts were prepared using TES buffer (1% SDS, 2 mM EDTA, 20 mM Tris–HCl pH 7.4), cytoplasmic and loosely held nuclear proteins (PN fraction) were prepared using CSK buffer (10 mM PIPES, 300 mM Sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA) and nuclear extracts were prepared using CSK buffer + 0.5% (V/V) Triton X-100.

Cell proliferation and apoptosis assays

Viable cells were counted 48 h post-transfection using trypan blue exclusion. Apoptotic cells were detected 24 h post-transfection in the absence and presence of 100 μM Z-VAD-FMK [*N*-Benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone] (Abcam) using Annexin V staining (10). Results were analysed for significance using the unpaired students *t*-test. After 24 h Annexin V FACs staining was performed as previously described.

Quantitative ChIP

For ChIP K562 cells (10^7 cells per ChIP) were transiently transfected with 5 μg of pMUG1-Myc-PRH expression

vector per chromatin preparation. ChIP was carried out as described in (17) with the following modifications. After cross linking cells were re-suspended in lysis buffer [50 mM Tris–Cl pH 8, 10 mM EDTA, 1% SDS, 1 mM PMSF, protease inhibitor cocktail tablet (Roche)] and divided into 5×10^6 fractions for sonication. Lysates were sonicated in a Biorupter (Diagenode) for 10 min at 4°C on medium power. For assessment of sonication efficiency, 10% of each sonicated chromatin lysate was incubated with proteinase K at 68°C for 2 h. DNA was then purified by phenol/chloroform extraction and ethanol precipitation and assessed for fragment size distribution by electrophoresis on a 1.5% agarose gel. For ChIP, 25 µg of chromatin lysate was incubated with Protein A magnetic beads (Dynabeads Invitrogen) and Myc antibody (9E10 NE Biolabs) or IgG antibody (Invitrogen) for 16 h at 4°C. Beads were collected with a magnet and then washed twice in wash buffer (10 mM Tris–Cl pH 7.5, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% Na–deoxycholate, 140 mM NaCl), twice in wash buffer with 500 mM NaCl, twice in wash buffer with 0.5% NP40 and twice in TE. DNA was eluted using elution buffer (20 mM Tris–Cl pH 7.5, 5 mM EDTA, 50 mM NaCl, with 1% SDS). After treatment with Proteinase K for 2 h at 68°C the DNA was purified by phenol/chloroform extraction and ethanol precipitation.

Quantitative PCR (qPCR) with the primers shown below was used to determine the relative amount of binding across the Vegfr-1 promoter. qPCR specific controls: a calibration line, melting curve analysis and the no-template control were performed for each primer pair. Equal concentrations of input and immunoprecipitated DNA were used for qPCR. To find the normalized signal ratio, the relative level of a genomic Vegfr-1 sequence was compared to the relative level of a genomic Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) sequence, as the Gapdh promoter is known not to be regulated by PRH. From this normalized signal ratio an enrichment ratio was calculated by comparing the normalized signal ratio for the immunoprecipitated DNA to the normalized signal ratio for the input DNA. IgG immunoprecipitated DNA was compared to input DNA in a similar fashion.

qPCR reactions were performed using a Rotogene RG-3000 (Corbett) with SYBR green master mix (Bioline). Reactions were incubated at 95°C for 10 min before 40 cycles of 95°C for 10 s, 60°C for 20 s, 72°C for 5 s.

Vegfr-1 primers pairs:

P5 R1 −7500 forward 5′-CTTCTCCGTGCTACTTCTTTCTGC-3′ reverse 5′-GGCCAGAGCTTTGGTTTCACTGAT-3′

P4 LM V forward 5′-TAGCTGAGACTACAGGCACAC-3′ reverse 5′-CTCTGGTTAGCAGTTCAGGGA-3′

P3 LM III forward 5′-ACGCTGCTCTTCCCACCTGAA-3′ reverse 5′-ACGCTCCAGAAATTCCATCCAGCA-3′

P2 R1 c3 forward 5′-CCTTGGTGTGCAGCCCAGAAATG-3′ reverse 5′-TAGTCCTATTGGAACCCGTCAGAG-3′

P1 R1 c2 forward 5′-GATTACCCGGGGAAGTGGTTGTCT-3′ reverse 5′-CCCCAGCCGCGCCTCACCTGT-3′

P+1 R1 +1700 forward 5′-TCTTTCTCAGTGGGAAGACTAACTAGACC-3′ reverse 5′-GTTATGTTAGCACCTTTCCCAACCTACAG-3′

GAPDH genomic primer pair:
Forward 5′-ACCCCTTTCACCATTAGGGACCTT-3′ reverse 5′-AGCCTGCCTGGTGATAATCTTTGC-3′

Quantitative reverse transcriptase-mediated PCR

RNA was produced following standard protocols and analysed as described previously (10). The qPCR was performed in triplicate using the primers listed below, and the data were analysed using Rotorgene 6 software (Corbett Research; Rotorgene RG-3000). Gapdh mRNA was used as an internal control.

qRT–PCR primer pairs:
Vegfr-1 forward 5′-TGGCCATCACTAAGGAGCACTCC-3′ reverse 5′-GGAACTGCTGATGGCCACTGTG-3′

Vegf forward 5′-ATCAGCGCAGCTACTGCCATCC-3′ reverse 5′-TCTCCTATGTGCTGGCCTTGGTG-3′

GAPDH forward 5′-TGATGACATCAAGAAGGTGGTGAAG-3′ reverse 5′-TCCTTGGAGGCCATGTGGGCCAT-3′

RESULTS

CK2 activity alleviates the repression of VSP genes by PRH

K562 cells are a CML cell line that express VEGF, VEGFR-1 and PRH. Chromatin immunoprecipitation (ChIP) assays have shown that PRH can bind directly to the Vegf and Vegfr-1 promoters in these cells (10). Knockdown of PRH in K562 cells results in the de-repression of Vegf and Vegfr-1 and, conversely, a 2- to 3-fold over-expression of PRH represses transcription of these genes (10). Although phosphorylation of PRH by CK2 can inhibit the binding of PRH to DNA, the degree to which this inhibits the repression of Vegf and Vegfr-1 transcription by PRH is not known. To determine whether increased CK2 activity alleviates the repression of these genes by PRH we over-expressed CK2 and PRH in K562 cells. We transiently transfected K562 cells with either an empty vector control (EVC), PRH expression vector alone, or with expression vectors for PRH and CK2. This results in a modest 2- to 3-fold over-expression of PRH (10). After 48 h Vegfr-1 mRNA levels were measured using quantitative PCR (qPCR). Over-expression of PRH causes a decrease in Vegfr-1 mRNA levels to ~30% of the unrepressed value (Figure 1A). However, in the presence of co-expressed CK2α and CK2β, Vegfr-1 mRNA levels are only weakly repressed (Figure 1A). CK2β alone fails to block repression by PRH as does CK2β and a kinase inactive K2α mutant (CK2αK68M). Similar results were obtained using qPCR to measure Vegf mRNA levels (Figure 1B).

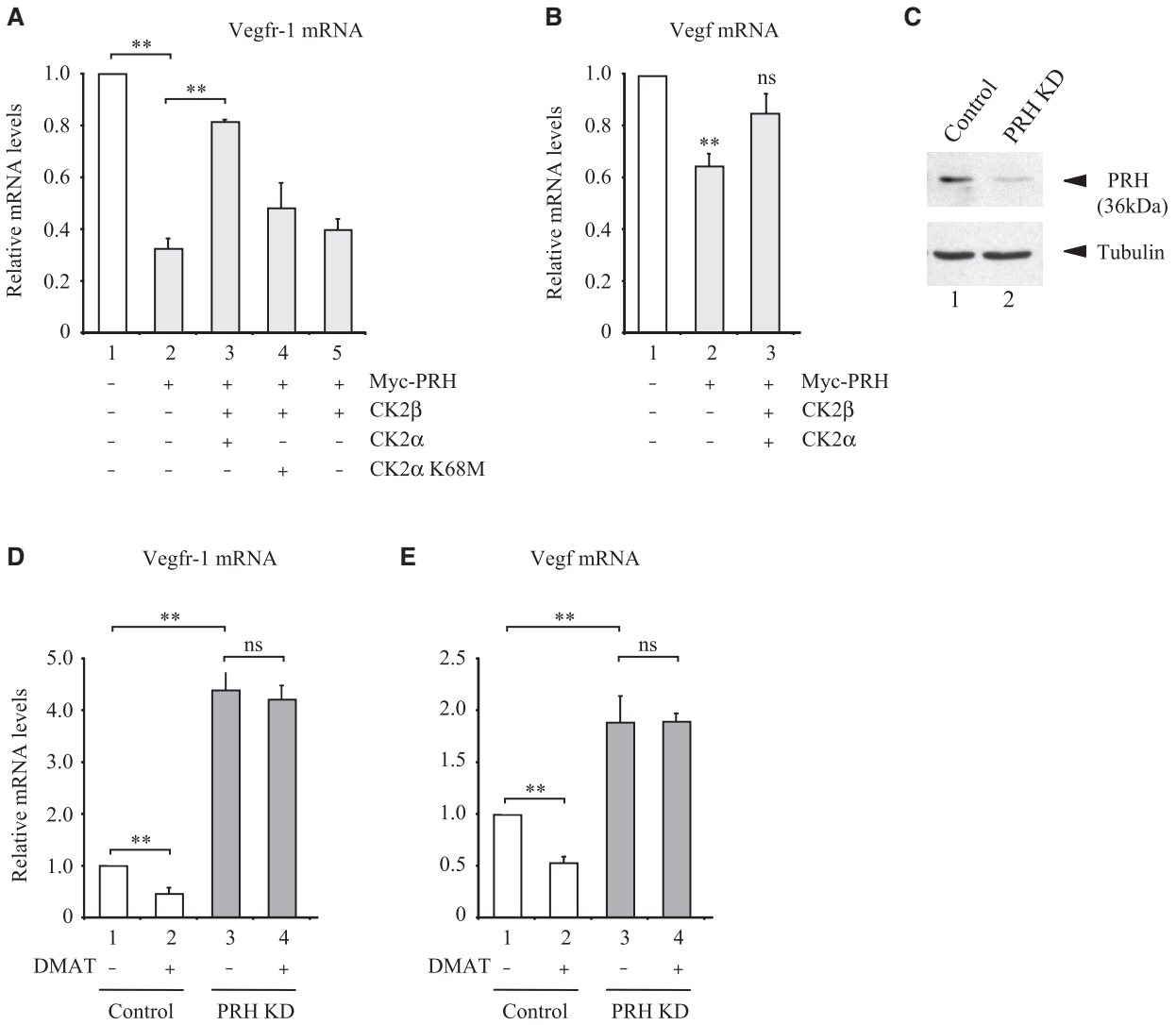

Figure 1. CK2 alleviates the repression of VSP genes by PRH. (**A**) Vegfr-1 mRNA levels in K562 cells 48 h post-transfection with empty vector (1), plasmid expressing Myc-PRH (5 μg) (2) or plasmids expressing Myc-PRH (5 μg) and HA-CK2 subunits (3 μg each) (3–5). mRNA levels were determined by qPCR and compared to Gapdh. Mean and standard deviation (M + SD), $n = 5$. ** indicates $P < 0.005$, ns indicates not significant. (**B**) Vegf mRNA levels in K562 cells transfected as in (A) determined as above. M + SD, $n = 5$. (**C**) PRH protein levels after transfection with scrambled vector control (SVC)(1) or PRH shRNA (2) followed by selection using puromycin. Endogenous PRH was detected using PRH-specific antibodies. (**D**) Vegfr-1 mRNA levels after PRH KD. Control and PRH KD cells were treated with 80 μM DMAT for 24 h prior to mRNA isolation. White bars represent SVC shRNA targeted control cells and grey bars PRH shRNA targeted cells. M + SD, $n = 3$. **$P < 0.005$, ns—not significant. (**E**) Vegf mRNA levels in the cells from (D).

Over-expression of CK2 has no effect on PRH protein levels (Figure 6C). We conclude that repression of the Vegfr-1 and Vegf genes by over-expressed PRH is reversed by over-expression of CK2. Over-expression of CK2 subunits in the absence of PRH has no effect of the expression of either Vegfr-1 or Vegf (Supplementary Figure S1). This suggests that endogenous PRH is maximally phosphorylated, either because it is tightly bound to DNA, or because it is present in other complexes that block phosphorylation.

Incubation of K562 cells with the CK2 inhibitor DMAT significantly decreases the amount of pPRH (20). To confirm that phosphorylation by CK2 antagonizes the repression of Vegf and Vegfr-1 by PRH we made use of PRH KD cells. Control cells and PRH KD cells (Figure 1C) were incubated with DMAT or left untreated and after 24 h Vegfr-1 and Vegf mRNA levels were determined using qPCR. In the control cells Vegfr-1 mRNA levels are significantly lower in the presence of DMAT (Figure 1D, 1 and 2). As expected, Vegfr-1 mRNA levels are much higher in PRH KD cells than in the control cells (Figure 1D, 3). However, in PRH KD cells treatment with DMAT fails to decrease Vegfr-1 mRNA levels (3 and 4). Very similar results were obtained using qPCR to measure Vegf mRNA levels (Figure 1E). Thus DMAT treatment results in increased repression of Vegfr-1 and Vegf only when PRH is present.

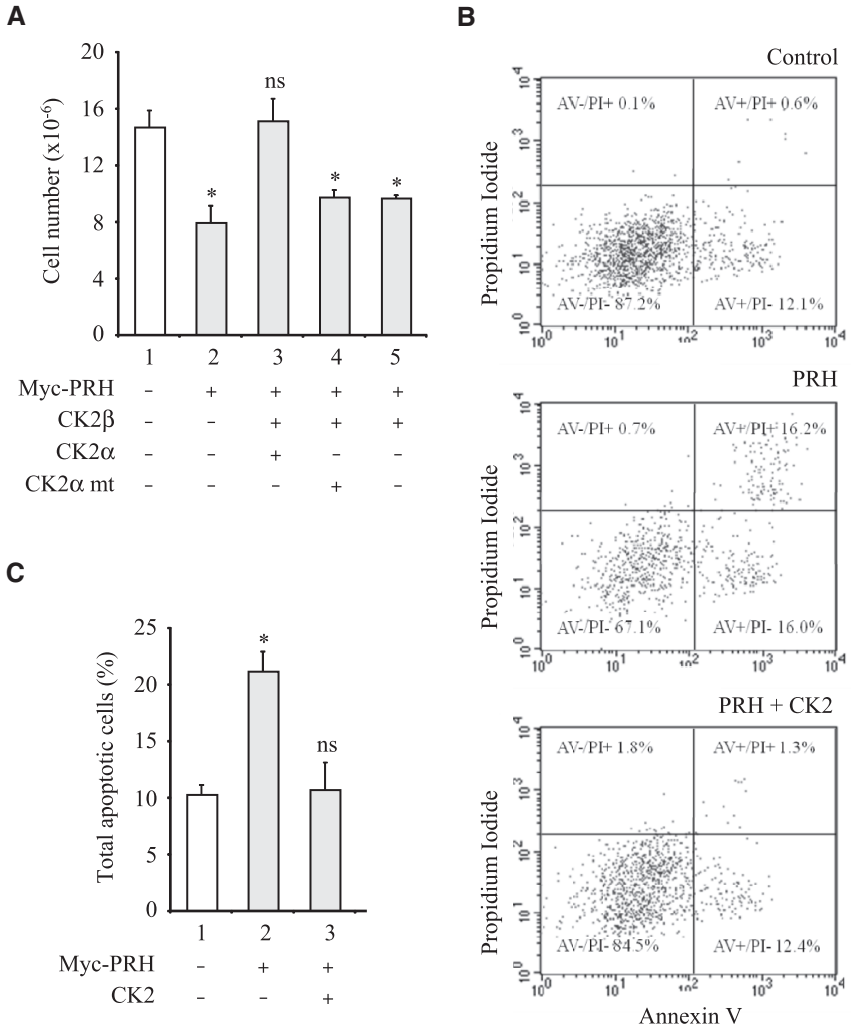

Figure 2. CK2 alleviates the inhibition of cell proliferation by PRH. (**A**) K562 cells were transfected with plasmids expressing Myc-PRH (5 μg) or Myc-PRH (5 μg) and CK2 subunits (3 μg each). Seventy-two hours post-transfection cells were counted using trypan blue exclusion. M + SD, $n = 5$. * indicates $P < 0.02$, ns—not significant. (**B**) K562 cells were transfected with 1 μg pEGFP alone (control), 1 μg pEGFP and 5 μg pMUG1-Myc-PRH (PRH) or 1 μg pEGFP, 5 μg pMUG1-Myc-PRH and 3 μg of plasmids expressing CK2α and CK2β. The cells were dual stained with propidium iodide (P.I.)/Annexin V (AV) (APC antibody) 24 h post-transfection and analysed by flow cytometry. Cells stained with both dyes are in late apoptosis. Cells stained for annexin V alone are in early apoptosis. The dot plot shows the percentage of live cells (PI−/AV−), necrotic cells (PI+), early apoptotic cells (AV+) and late apoptotic cells (AV+/PI+) after gating for GFP+ cells. Representative data. (**C**) The experiment shown in (C) was repeated three times and the graph shows the percentage of early and late apoptotic cells. * indicates $P < 0.02$, ns—not significant.

CK2 antagonizes the inhibition of cell proliferation by PRH

To determine whether the inhibitory effects of PRH on cell proliferation are antagonized by CK2, we expressed PRH alone, or PRH and CK2, in K562 cells and monitored cell number 72 h post-transfection. Although only 35–50% of the cells are transfected under these conditions, over-expression of PRH significantly decreases cell number (Figure 2A, 2). Co-expression with CK2α and CK2β abrogates the effect of PRH on cell number (3). In contrast, co-expression with kinase inactive CK2α (K68M) and wild-type CK2β, or the CK2β subunit alone does not reverse the effects of PRH over-expression (4 and 5). Furthermore, over-expression of CK2 alone has no effect on cell number (Supplementary Figure S2).

We have shown previously that over-expression of PRH in K562 cells can induce apoptosis and that this can be abrogated by over-expressing VEGF-signalling proteins from PRH-independent reporters (10). To confirm that the pro-apoptotic activity of PRH in these cells is blocked by CK2, the survival of the transfected cells was measured by expressing eGFP with PRH or eGFP with PRH and CK2 and staining the cells with propidium iodide (for DNA) and APC-Annexin V (for apoptosis). Flow cytometry shows that over-expression of PRH results in >10-fold increase in the number of cells in late apoptosis 24 h post-transfection and a more modest increase in early apoptosis (Figure 2B, middle). In contrast, over-expression of PRH and CK2 has very little effect on the number of cells in apoptosis (Figure 2B, bottom and Figure 2C). As expected,

treatment with an inhibitor of apoptosis (Z-VAD-FMK) blocks PRH-induced apoptosis (Supplementary Figure S3A). However, the inhibition of PRH-induced apoptosis has no effect on the down-regulation of Vegfr-1 mRNA levels by PRH (Supplementary Figure S3). We conclude that CK2 antagonizes the effect of PRH on apoptosis and that this results in increased cell survival.

CK2 blocks the binding of PRH to the Vegfr-1 promoter

To examine the effects of PRH phosphorylation on VSP gene expression and cell survival in more detail, we looked at the effects of two mutated PRH proteins. We introduced serine to cysteine mutations at the positions known to be phosphorylated by CK2 to produce PRH S163C/S177C (PRH CC). These mutations prevent phosphorylation by CK2 at these positions. The PRH S163E/S177E (PRH EE) protein carries mutations of serine to glutamate at the same positions and has been described previously (20). The PRH EE phosphomimic is unable to bind DNA (20). As expected, wild-type PRH represses Vegfr-1 and CK2 over-expression counteracts this repression (Figure 3A, 2 and 3). PRH CC also represses Vegfr-1 mRNA levels but CK2 is unable to counteract this repression (Figure 3A, 4 and 5). In contrast, PRH EE is unable to decrease Vegfr-1 expression and CK2 has no effect in the presence of this protein (Figure 3A, 6 and 7). Western analysis confirms these proteins are expressed at equivalent levels (Figure 3B).

To show that the differential effects of CK2 on PRH- and PRH CC-mediated repression of Vegfr-1 gene expression are due to changes in DNA binding, we performed quantitative ChIP assays. Chromatin obtained from cells expressing PRH and the mutant PRH proteins described above was sheared by sonication using conditions that reproducibly produce an average fragment size of <400 bp (Figure 3C). Figure 3D (upper panel) shows a cartoon of the Vegfr-1 promoter and the positions of multiple PRH-binding sites that we have identified previously (10). Quantitative ChIP shows that PRH binds to Vegfr-1 promoter sequences extending from ~−5600 bp 5′ and +1700 bp 3′ relative to the first exon (Figure 3D, 1). This binding is not likely to be an artefact of over-expression because we have shown that PRH is only moderately over-expressed relative to endogenous PRH in these cells (10). While the possibility that chromatin fragments >400 bp result in ChIP at locations distal to the PRH-binding sites cannot be completely excluded, the ability of PRH to bind to degenerate sequences (10) and to oligomerize and wrap extensive DNA sequences (17), suggests that it is more likely that PRH binds at multiple positions across an extended region of the Vegfr-1 promoter. There is no enrichment for PRH in the presence of non-specific IgG antibody. Importantly PRH binding across the whole promoter region is lost when PRH and CK2 are co-expressed (Figure 3D, 2). In contrast, PRH CC binding at the Vegfr-1 promoter is not blocked by CK2 (Figure 3D, 3 and 4). As expected, PRH EE does not bind to the promoter (data not shown). Quantitative ChIP shows that the differential regulation of PRH and PRH CC by CK2 occurs across an extended region of genomic DNA that is known to be involved in regulation of the Vegfr-1 gene. We infer that CK2 will have a profound effect on Vegfr-1 gene expression since phosphorylation of PRH will allow many activators and epigenetic modulators access to the promoter.

To examine whether the mutated PRH proteins regulate cell proliferation, K562 cells expressing each protein were monitored for cell number and apoptotic cell death as above. Over-expression of PRH brings about decreased cell number and this is blocked by CK2 over-expression (Figure 4A). In contrast, PRH CC decreases cell number but the effects of this protein are not counteracted by CK2. As expected, PRH EE is unable to inhibit cell number. CK2 co-expression also counteracts PRH-induced apoptosis (Figure 4B, 2 and 3) but it does not counteract the pro-apoptotic effect of PRH CC (Figure 4B, 4 and 5). These data strongly support the hypothesis that phosphorylation of PRH by CK2 abolishes direct transcriptional repression of VSP genes and that this in turn results in increased cell survival.

CK2 alters the localization of PRH

We have shown that phosphorylation by CK2 blocks the DNA-binding activity of PRH (20). We wondered whether phosphorylation might also influence the localization of this protein. PRH, PRH EE and PRH CC are all present in the nucleus in immunostaining experiments suggesting that phosphorylation does not affect nuclear localization (Figure 5B). However, to examine the sub-nuclear localization of these proteins we made use of biochemical (Figure 5A and C) and *in situ* sub-cellular fractionation (Figure 5A). Cells were fractionated into cytoplasmic and loosely held nuclear proteins [the Post-Nuclear (PN) fraction] and tightly held nuclear proteins [Nuclear (N) fraction]. PRH and PRH CC are present in the PN and N fractions whereas PRH EE is present predominantly in the PN fraction (Figure 5A). Antibodies against Lamin A/C and Tubulin were used to verify successful fractionation and equal loading. The difference in localization was confirmed by *in situ* fractionation of cells growing on coverslips (Figure 5B). While PRH and PRH CC are tightly held in the nucleus (Figure 5A, rows 4 and 5), PRH EE is readily depleted from the nucleus following removal of the cytoplasmic and loosely held nuclear proteins. Moreover, over-expression of CK2 results in the loss of co-expressed Myc-PRH from the nuclear fraction (Figure 5C), further suggesting that phosphorylation alters PRH localization.

CK2 alters PRH stability

To compare the stability of hypophosphorylated PRH (hypo-PRH) and pPRH we incubated K562 cells with the translation inhibitor anisomycin and performed fractionation and western blotting using phosphospecific PRH antibodies (20). Endogenous hypo-PRH is present in both the PN and N compartments (Figure 6A, top, 1 and 5). pPRH is also present in both fractions, although it is more prominent in the PN fraction (second top panel, 1 and 5) confirming the observations made with PRH EE above. Hypo-PRH is stable over 24 h in both fractions whereas

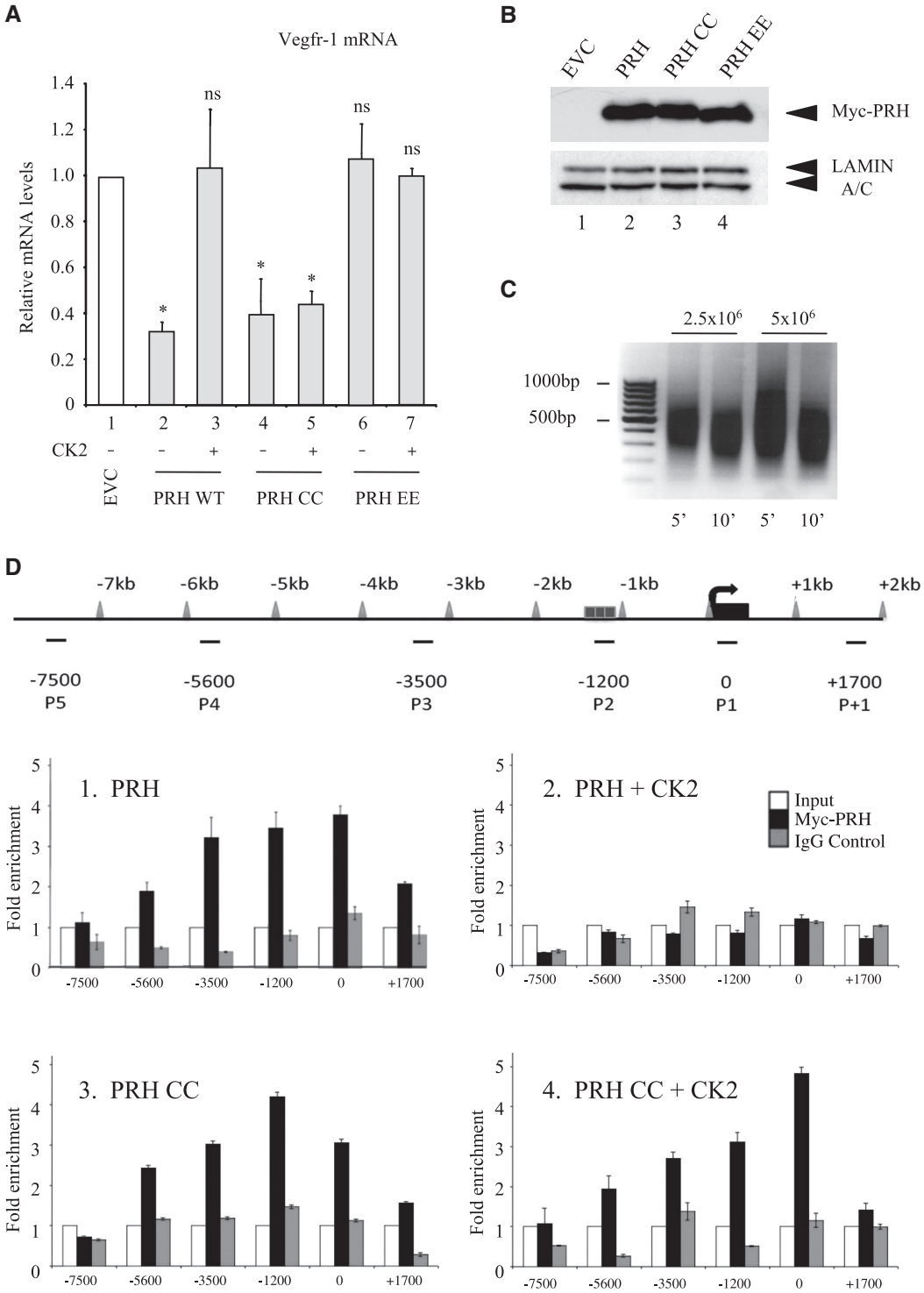

Figure 3. CK2 abolishes DNA binding and the repression of VSP genes by PRH. (**A**) Vegfr-1 mRNA levels in K562 cells 48 h post-transfection with an EVC or plasmids expressing PRH, PRH CC and PRH EE either alone, or in conjunction with plasmids expressing CK2 subunits. mRNA levels were determined as in Figure 1. M + SD, $n = 3$. $^*P < 0.05$, ns—not significant. (**B**) Western blot of whole-cell extracts from K562 cells transfected as in (A). (**C**) K562 cells were transfected as in (A) and chromatin was assessed for distribution of fragment size by electrophoresis on a 1% agarose gel. M = 1 kb Marker, chromatin from 2.5×10^6 cells sonicated for 5 min (1) or 10 min (2), chromatin from 5×10^6 cells sonicated for 5 min (3) or 10 min (4). Sonication of chromatin from 5×10^6 cells results in fragments averaging <400 bp and these conditions were used in ChIP. (**D**) Upper panel—a cartoon of the Vegfr-1 genomic region, showing relative positions of the Vegfr-1 promoter (bent arrow), clusters of PRH-binding sites (filled boxes), and Vegfr-1 primer sequences used for ChIP. Lower panels- enrichment of Myc-PRH proteins bound Vegfr-1 primer sequences relative to input. Template DNA was precipitated using the Myc 9E10 antibody or IgG. M + SD, $n = 4$.

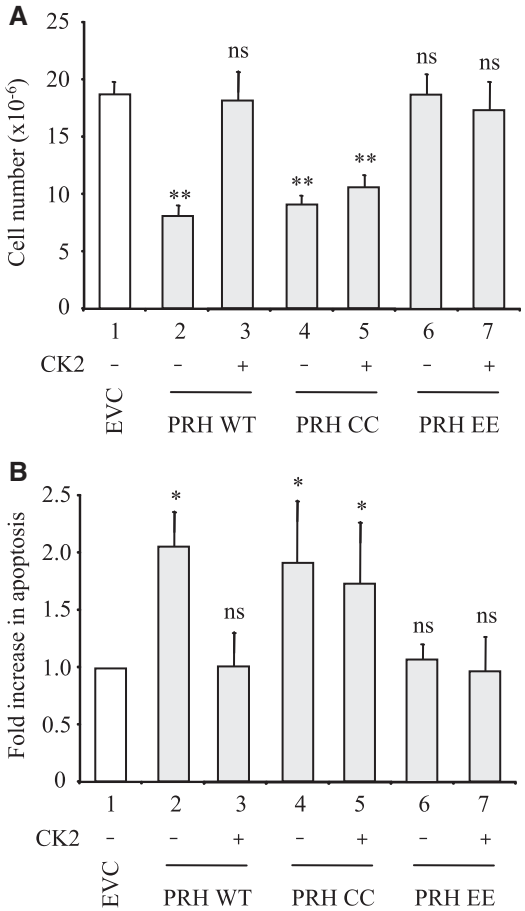

Figure 4. CK2 reverses the inhibition of cell proliferation by PRH but not PRH CC. (**A**) K562 cells were transfected as in Figure 3A and 72 h post-transfection cells were counted as in Figure 1. M + SD, $n = 5$. $^{**}P < 0.005$, ns—not significant. (**B**) K562 cells were co-transfected with a plasmid expressing GFP and an empty vector (EVC) or plasmids expressing GFP, PRH proteins and CK2 subunits. Twenty-four hours post-transfection the cells were dual stained as in Figure 2. The graph shows the fold change in the number of apoptotic cells (AV+/PI+) after gating for GFP+ cells. M + SD, $n = 3$. $^{*}P < 0.05$, ns—not significant.

pPRH is unstable (compare lanes 1 and 4 in both panels). To determine whether endogenous pPRH is a substrate for the proteasome we incubated cells with anisomycin and the proteasome inhibitor MG132. In the presence of both inhibitors pPRH is stabilized in both fractions (Figure 6B second panel). Incubation of cells with the proteasome inhibitor Lactacystin also showed the same result (data not shown). However, there is little change in the amount of hypo-PRH in either fraction since the protein is stable over this time period and anisomycin treatment blocks further protein synthesis (Figure 6B, top panel). We conclude that pPRH is a substrate for the proteasome.

Interestingly, the phosphospecific PRH antibodies detect an endogenous protein with an apparent molecular weight of 27 kDa as well as the band corresponding to endogenous pPRH (Figure 6A and B). A protein that migrates with a marginally greater apparent molecular weight is detected using the Myc 9E10 antibody in cells expressing Myc-tagged PRH (Figure 6C, 2). Furthermore, the amount of this protein is increased in cells co-expressing Myc-PRH and CK2 (Figure 6C, 3). These data suggest that the endogenous 27-kDa protein is a stable truncated PRH protein produced following the cleavage of pPRH by the proteasome. Since a protein corresponding to this cleavage product is detected by the Myc antibody when the N-terminally tagged Myc-PRH protein is expressed in cells, we conclude that the cleavage event must remove the C-terminal region of the PRH protein. The apparent size of the truncated protein suggests that cleavage of pPRH removes the entire C-terminal domain (amino acids 211–277) leaving the intact N-terminal transcription repression domain and central PRH homeodomain; we will call this truncated protein PRHΔC.

To confirm that phosphorylation of PRH by CK2 is required for the production of PRHΔC, we expressed Myc tagged wild-type PRH, PRH EE phosphomimic and the PRH CC protein that is unable to be phosphorylated by CK2 in K562 cells and used western blotting to examine whether the truncated protein is produced in each case. As expected based on the experiments described above, when wild-type is expressed in these cells PRHΔC is detectable using the Myc antibody (Figure 6D, 2). Interestingly, the Myc tagged PRHΔC protein is present at higher levels in cells expressing the PRH EE phosphomimic (Figure 6D, 3 and Figure 6E). Furthermore, Myc tagged PRHΔC is not detectable in cells expressing PRH CC (Figure 6D, 4). These data show that phosphorylation at these sites is required for the production of PRHΔC. Like the full-length hypo-PRH protein, the PRHΔC protein is stable in the presence of anisomycin (Figure 6A). However, PRHΔC is predominantly present in the PN fraction whereas full-length hypo-PRH is present in both the PN and N fractions. This is consistent with localization of PRH EE to the PN fraction (Figure 5B) and further suggests that PRHΔC is phosphorylated.

We conclude that in addition to inhibiting the DNA-binding activity of PRH phosphorylation by CK2 decreases the nuclear association of this protein and brings about the cleavage of PRH by the proteasome resulting in the accumulation of a truncated PRH cleavage product.

PRHΔC is a transdominant negative regulator of PRH

Since the PRHΔC protein appears to be stable, accumulating to high levels in the presence of anisomycin relative to pPRH, we wondered whether this processed fragment might have an effect on the ability of hypo-PRH to regulate transcription. To test this we created a truncated version of PRH EE in which we deleted the C-terminal domain from amino acids 211 to 277 (PRHΔC EE). We then examined the effect of this truncated construct on the ability of PRH to repress transcription of the endogenous Vegfr-1 gene. As expected, a modest over-expression of PRH results in the repression of Vegfr-1 mRNA levels (Figure 6F, 2). Also as expected, the PRHΔC EE protein fails to repress Vegfr-1 mRNA levels (Figure 6F, 3). Interestingly, co-transfection of the PRH

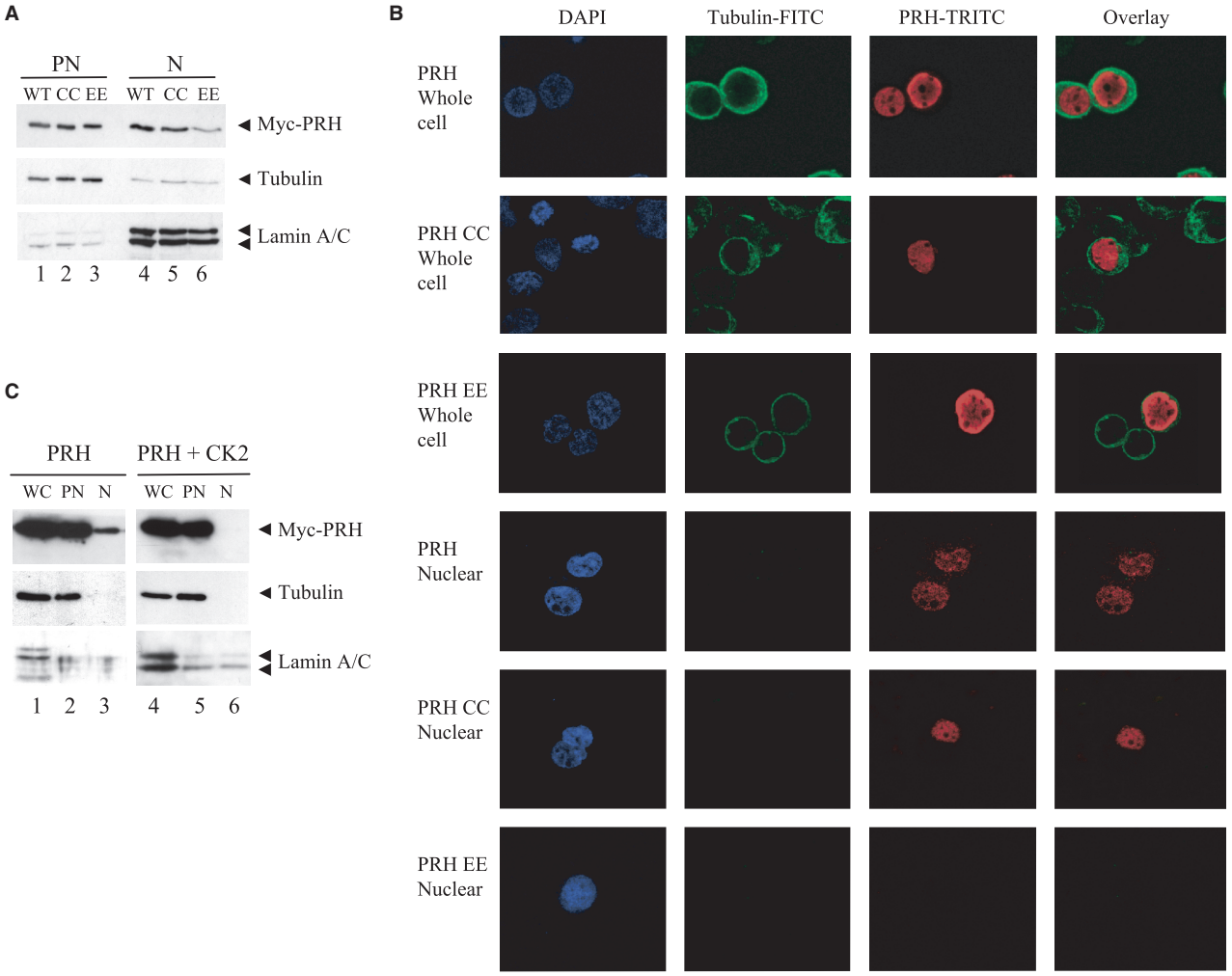

Figure 5. A PRH phosphomimic shows altered intracellular localization. (**A**) K562 cells were transfected with vectors expressing Myc-tagged PRH, PRH CC or PRH EE and then fractionated into cytoplasmic and loosely held nuclear proteins (PN) and tightly held nuclear proteins (N). The extracts were western blotted for PRH using the Myc antibody (top panel). The blot was stripped and reprobed for Tubulin and Lamin A/C as controls for fractionation and loading. (**B**) K562 cells were transiently transfected as above and then adhered to polylysine coated coverslips. Top three rows, whole-cell images. Bottom three rows, cells treated with CSK buffer containing 0.1% SDS to remove cytoplasmic and loosely held nuclear proteins. DNA was stained with DAPI. Tubulin was visualized using an anti-Tubulin antibody and FITC-labelled secondary. PRH was visualized using the Myc 9E10 antibody and a TRITC-labelled secondary. Viewed using a Leica DM IRBE confocal microscope. (**C**) K562 cells were transfected with plasmids expressing Myc-PRH alone or Myc-PRH and HA-CK2 α and β subunits (as in Figure 1A). The cells were then fractionated into whole-cell extract (WC), PN and N fractions as in (part A above). The extracts were western blotted for PRH using the Myc antibody (top panel). Tubulin and Lamin A/C were used as controls for fractionation and loading.

expression vector with increasing amounts of the PRHΔC EE expression vector results in a dose-dependent reduction in the repression of Vegfr-1 mRNA levels by PRH (Figure 6F, 4–6). Expression of PRHΔC EE does not change the expression level of PRH in this experiment (Figure 6G). These data show that PRHΔC EE acts a transdominant negative regulator of the full-length PRH protein. This suggests that the endogenous PRHΔC protein produced by the proteosomal digestion of pPRH will also act as a transdominant negative regulator of PRH activity.

PRHΔC sequesters TLE co-repressor proteins

We have shown previously that PRH recruits members of the TLE family of co-repressor proteins in order to repress the transcription of its target genes (18). An Eh1 motif present in the N-terminal repression domain of PRH mediates binding to TLE proteins and an F32E mutation in this motif blocks both binding to TLE proteins and transcriptional co-repression (18,22). The PRHΔC EE protein carries the Eh1 motif and although this protein is unable to repress transcription we wondered whether it might bind and sequester TLE proteins and thereby act as transdominant negative for full-length PRH. To test this hypothesis we first expressed PRHΔC EE in K562 cells and performed co-immunoprecipitation assays for TLE. The PRHΔC EE protein is able to co-immunoprecipitate FLAG tagged TLE (Figure 7A) whereas this protein is not

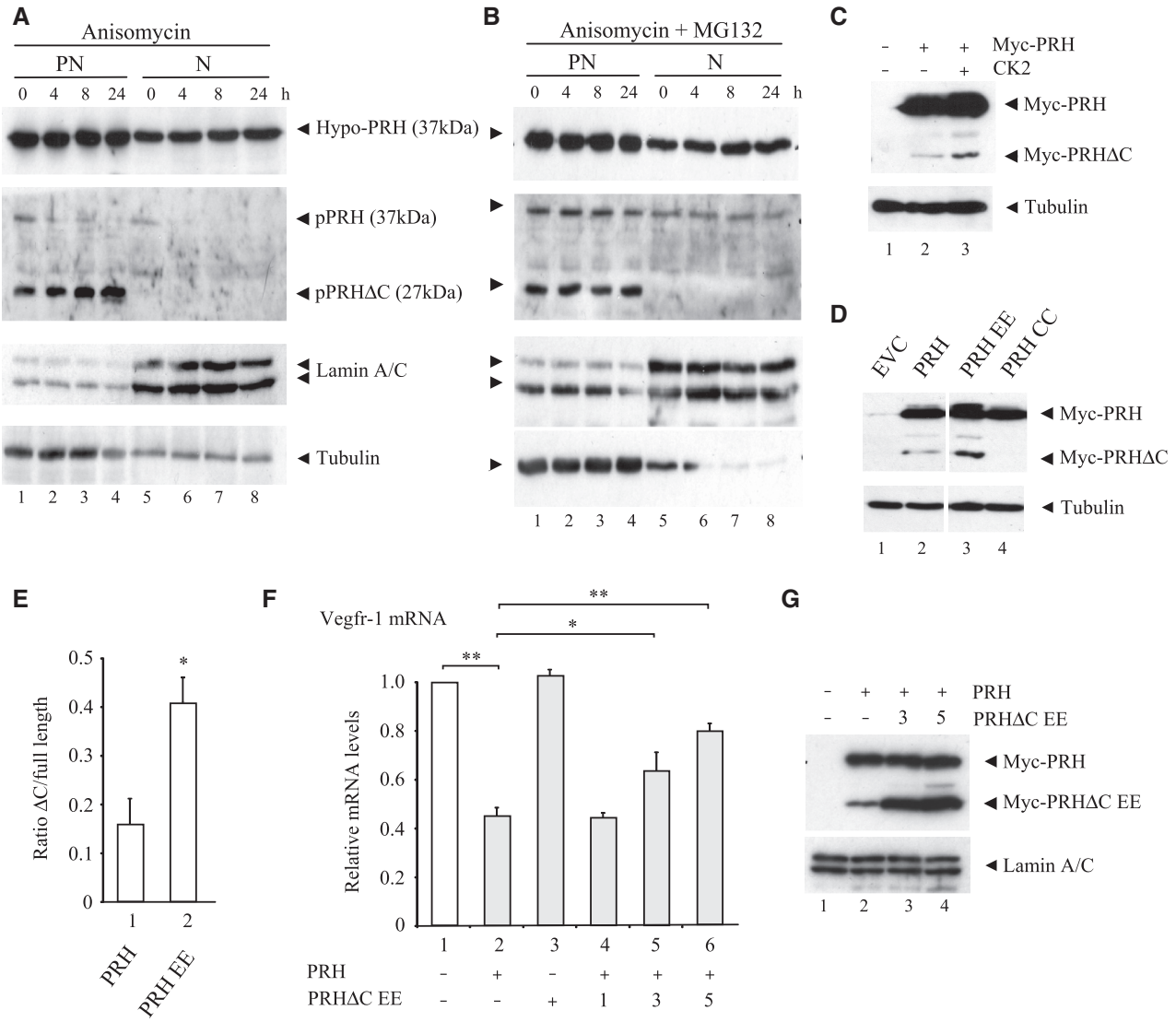

Figure 6. pPRH is rapidly cleaved by the proteasome to produce a stable product. (**A**) Untransfected K562 cells were treated with Anisomycin for the times indicated and then fractionated as in Figure 5. The extracts were western blotted for endogenous PRH using antibodies that recognize hypo-PRH or pPRH. The blot was stripped and reprobed for Lamin A/C and Tubulin as controls for fractionation and loading. (**B**) The experiment described in (A) was repeated using K562 cells treated with 40 μM anisomycin and the proteasome inhibitor MG132 (10 μM). (**C**) K562 cells were transiently transfected with plasmids expressing Myc-PRH (5 μg) or Myc-PRH (5 μg) and HA-CK2 subunits (3 μg each). Twenty-four hours post-transfection whole-cell extracts were western blotted for Myc-tagged proteins using the Myc9E10 antibody. The blot was stripped and reprobed for Tubulin as a control for loading. (**D**) K562 cells were transiently transfected with EVC or plasmids expressing Myc-PRH (5 μg), Myc-PRH EE (5 μg) and Myc-PRH CC (5 μg). Twenty-four hours post-transfection whole-cell extracts were western blotted for Myc-tagged proteins and Tubulin as a control for loading. (**E**) The ratio of PRHΔC to full-length PRH was determine from three independent experiments performed as in (D). $*P < 0.05$, (**F**) Vegfr-1 mRNA levels in K562 cells 48 h post-transfection with plasmids expressing Myc-PRH (5 μg) alone, Myc-PRHΔC EE (5 μg) alone or Myc-PRH (5 μg) and Myc-PRHΔC EE (1, 3 and 5 μg). mRNA levels were determined by qPCR and compared to Gapdh. Mean and standard deviation (M + SD), $n = 5$. $*P < 0.05$, $**P < 0.01$, ns—not significant. (**G**) Myc-PRH and Myc-PRHΔC EE protein levels in the experiment described in (F) were determined by western blotting. Lamin A/C was used as a control for loading.

co-immunoprecipitated by control antibodies. Furthermore, although PRHΔC EE and full-length PRH are expressed at equivalent levels, PRHΔC EE is able to co-immunoprecipitate FLAG-TLE much more robustly than full-length PRH (Figure 7A, compare lanes 2 and 5 top panel). This suggests that PRHΔC EE binds to TLE with higher affinity than PRH. To confirm the PRHΔC EE-TLE interaction and to show that endogenous PRHΔC binds to TLE, we performed a co-immunoprecipitation assay using FLAG-TLE as bait. Endogenous PRHΔC co-immunoprecipitates with FLAG-TLE but does not co-immunoprecipitate with control antibodies (Figure 7B).

To determine whether binding to TLE is important for the ability of PRHΔC to act as a transdominant negative protein we introduced the well-characterized F32E mutation into PRHΔC EE. The PRHΔC EE F32E mutant fails to act as a dominant negative for full-length PRH (Figure 7C) although the protein is expressed at equivalent levels to PRHΔC EE (Figure 7D).

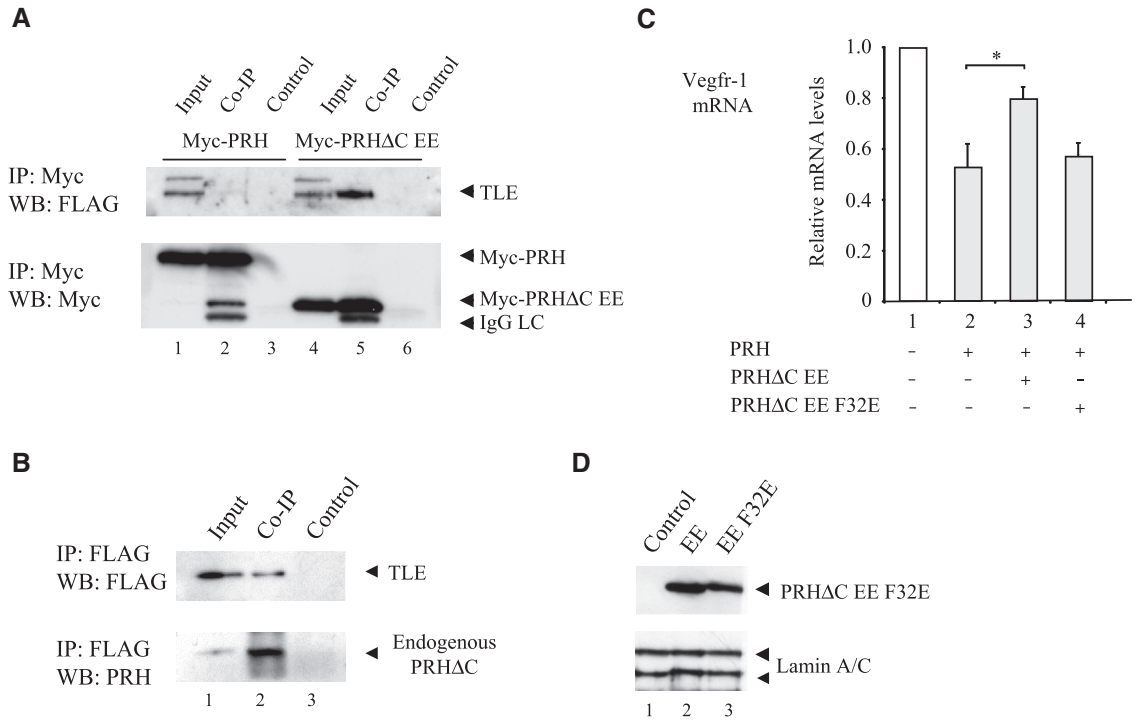

Figure 7. The transdominant negative activity of PRHΔC requires binding to TLE co-repressor proteins. (**A**) K562 cells were co-transfected with expression vectors for FLAG-TLE1 and Myc-PRH or FLAG-TLE1 and Myc-PRHΔC EE and nuclear extracts prepared for co-immunoprecipitation. The top panel shows a western blot for FLAG-TLE1 in the nuclear extract (1 and 4) and in the same extract after immunoprecipitation with the Myc9E10 antibody (2 and 5) or control rabbit IgG antibody (3 and 6). The blot was striped and reprobed with the Myc9E10 antibody to confirm expression of Myc-PRH and Myc-PRHΔC EE (bottom panel). The secondary antibody also picks up the mouse IgG light chain (IgG LC). (**B**) K562 cells were transfected with an expression vector for FLAG tagged TLE1 and nuclear extracts prepared for co-immunoprecipitation. The bottom panel shows a western blot for endogenous pPRH in the nuclear extract (1) and after immunoprecipitation with the FLAG antibody (2) or control mouse IgG antibody (3). (**C**) Vegfr-1 mRNA levels in K562 cells 48 h post-transfection with plasmids expressing Myc-PRH (5 μg) alone, Myc-PRH (5 μg) and Myc-PRHΔC EE (5 μg) or and Myc-PRH and Myc-PRHΔC EE F32E (5 μg). mRNA levels were determined by qPCR and compared to Gapdh. Mean and standard deviation (M + SD), $n = 3$. $^*P < 0.05$, (**D**) Cell extracts from (**C**) were used to determine the expression levels of Myc-PRHΔC EE and Myc-PRHΔC EE F32E using western blotting. The blot was stripped and reprobed for Lamin A/C as a loading control.

We conclude that PRHΔC sequesters TLE co-repressor proteins and that this is responsible for the ability of this protein to act a transdominant negative regulator of full-length PRH.

DISCUSSION

PRH is an oligomeric transcription factor that regulates the proliferation of multiple cell types in development. We have demonstrated previously that PRH also controls the proliferation of haematopoietic and breast tumour cells (10). One mechanism by which PRH can control cell proliferation is by regulating the transcription of multiple genes encoding components of the VSP. Here we have shown using quantitative ChIP that PRH can be immunoprecipitated at regions extending from 5600 bp 5′ to 1700 bp 3′ of the Vegfr-1 first exon and is most strongly associated with sequences ~1-kb upstream of the core Vegfr-1 promoter. Since PRH distorts and compacts long stretches of DNA *in vitro* (16,17) and binds to extended tandem arrays of repeated 5′-ATTAA-3′ sequences and related DNA sequences, we speculate that the presence of PRH across the Vegfr-1 promoter reflects the DNA-binding properties of the PRH oligomer. Presumably, the binding of PRH across extensive promoter sequences contributes to the repression of transcription by excluding multiple activator proteins. Here we have demonstrated that CK2 is able to antagonize PRH binding across the entire Vegfr-1 promoter alleviating PRH-mediated repression. It would seem likely that PRH represses other VSP genes in a similar manner and that CK2 abrogates PRH-mediated repression in each case. In keeping with this conclusion we have shown here that CK2 abrogates the repression of Vegf mRNA levels by PRH. Since PRH represses multiple VSP genes it is likely that phosphorylation of PRH by CK2 results in an increase in VEGF signalling. We have shown previously that in K562 cells PRH KD results in increased VEGF signalling and increased cell survival (10). In cells that express PRH, the interplay between PRH and CK2 and the consequent regulation of cell survival is likely to be important in allowing the appropriate level of cell proliferation in response to cues from the extracellular and intracellular environment.

In addition to blocking the DNA-binding activity of PRH, phosphorylation by CK2 decreases PRH nuclear association and targets this protein for cleavage by the proteasome. It would seem likely that all of these

mechanisms contribute to the ability of CK2 to antagonize the inhibition of cell proliferation by PRH. This suggests a model in which phosphorylation of PRH by CK2 leads to loss of DNA binding, loss of nuclear retention, increased PRH degradation and the production of a phosphorylated and truncated PRH protein which we have termed PRHΔC (HhexΔC). The PRHΔC protein then acts as a transdominant negative regulator of full-length PRH very likely via the sequestration of TLE co-repressor proteins. The production of the PRHΔC following the cleavage of pPRH would thus provide a very effective mechanism to switch off PRH in cells. The PRHΔC protein appears to be very stable in contrast to the rapidly degraded pPRH protein. The sustained phosphorylation of even a small percentage of PRH by CK2 would result in the accumulation of large amounts of PRHΔC that could down-regulate the activity of unphosphorylated PRH. This novel regulatory mechanism implies that small changes in CK2 activity and the overall level of PRH phosphorylation could have dramatic effects on the ability of PRH to regulate transcription. TLE proteins are recruited by many other transcription factors to facilitate the regulation of diverse genes and it is likely that PRHΔC will also regulate some or all of these targets.

Finally it is important to point out that these experiments suggest a molecular rationale for the use of CK2 inhibitors in the treatment of primary CML. The restoration of PRH activity through inhibition of CK2 may be particularly of value in Imatinib or Dasatinib resistant CML or in targeting the quiescent cancer stem cells in CML which are less dependent on BCR-ABL activity (24). These data also support a role for PRH as a tumour suppressor gene in haematopoietic myeloid lineages.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–3.

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